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Effects of Amiloride and Ouabain on Contractile State, Ca and Na Fluxes, and Na Content in Cultured Chick Heart Cells

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SUMMARY

Amiloride has been reported to reduce the positive inotropic and toxic actions of cardiac glycosides in patients as well as in experimental animals. To investigate the mechanism by which amiloride interacts with glycosides at the cellular level, we examined the effect of amiloride and ouabain on cellular Na content and uptake, Ca flux via Na-Ca exchange, and contractile state. Amiloride (1 mM) reduced cellular Na content by 16% ($p < 0.05$) under normal conditions and by 45% in the presence of 1 mM ouabain compared to respective control values observed in the absence of amiloride. Amiloride (1 mM) reduced the initial rate of ^{45}Ca uptake by 40% in ouabain (1 mM)-treated cells. This reduction of ^{45}Ca uptake could be mimicked by lowering cellular Na content by 42%. Amiloride (1 mM) did not alter significantly the initial rate of ^{24}Na uptake under normal conditions but reduced it by 32% in the presence of 3 μM ouabain. Amiloride (1 mM) produced a transient increase followed by a gradual decrease in the amplitude of cell motion over 60 min to 10% of control level. At other concentrations between 0.1 and 3 mM, amiloride produced negative inotropic effects only. Amiloride increased the concentration of ouabain needed to produce rhythm disturbances and contracture, and reduced Na-free contracture amplitude by 18%. These results are consistent with the view that amiloride antagonizes the arrhythmogenic effects of ouabain by inhibiting the glycoside-induced elevation in cellular Na content and, consequently, the increases in $[Ca]_i$ that occur via Na-dependent pathways. The reduced cellular Na content appears to be due to decreased Na influx via Na-H exchange.

Amiloride is a widely used potassium-sparing diuretic drug that has been reported to reduce digitalis sensitivity of the heart by a direct cardiac action (1–3) as well as by altering the pharmacokinetics of digoxin (4, 5). Thus, amiloride protected the heart against toxic effects of digitalis, delaying the onset of rhythm disturbances and cardiac arrest (2). Studies using isolated guinea pig heart preparations also demonstrated that amiloride antagonized the arrhythmogenic actions of cardiac glycosides (6, 7). The amiloride-induced lengthening of action potential duration in canine Purkinje fibers (8), in isolated papillary muscle of guinea pig heart, or in Purkinje fiber of dog heart (3) also suggests that amiloride may shift the glycoside-induced shortening of the refractory period in the opposite direction, thereby opposing the direct action of digitalis on the heart. These results indicate that the direct action of amiloride on the heart may be an important mechanism underlying the reduced sensitivity to toxic effects of digitalis.

Several studies have attempted to elucidate the mechanism underlying the amiloride-digitalis interaction at the cellular level. Amiloride caused inhibition of Na-Ca exchange in beef heart sarcolemmal vesicles (6), in erythroleukemia cells (9), and in rat brain synaptosomes (10). Since digitalis augments $[Ca]_i$ via Na-Ca exchange to exert its cardiac effects (11, 12), the ability of amiloride to inhibit Na-Ca exchange led to the suggestion that amiloride reduces digitalis sensitivity of the heart by inhibition of this exchange carrier (6, 13). However, amiloride is also known to be a potent inhibitor of Na-H exchange in cultured heart cells (14, 15) and sheep cardiac Purkinje fibers (16), as well as in many other cell types (17, 18). These findings led Frelin *et al.* (14) to suggest that amiloride antagonized the effect of ouabain by reducing Na influx via Na-H exchange. Thus, the cellular mechanism by which amiloride reduces the effects of digitalis on the heart remains uncertain. To address the cellular mechanism involved in the amiloride-digitalis interaction, we investigated the effects of amiloride and ouabain alone and in combination on contractile state, Na-dependent and total Ca fluxes, Na influx, and cellular Na content using spontaneously contracting cultured chick ventricular cells.

Materials and Methods

Tissue culture. Monolayer cultures of spontaneously contracting chick embryo ventricular cells were prepared as previously described (19). Briefly, hearts of 10-day-old chick embryos were removed and placed in Ca^{2+}- and Mg^{2+}-free Hanks' solution (Gibco Laboratories, Grand Island, NY). Ventricular muscles were cut into small fragments

ABBREVIATIONS: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TTX, tetrodotoxin.

(less than 0.5 mm^3), and individual cells were isolated by trypsinization with 0.025% (w/v) trypsin at 37°. Cell suspensions were centrifuged and the resulting pellet was resuspended in culture medium containing 6% heat-inactivated fetal calf serum, 40% M199 with Hanks' salt, 0.1% penicillin-streptomycin solution, and 54% balanced salt solution. Balanced salt solution contained 116 mM NaCl, 1.0 mM NaH_2PO_4, 0.8 mM $MgSO_4 \cdot H_2O$, 1.18 mM KCl, 26.2 mM $NaHCO_3$, 0.87 mM $CaCl_2$, and 5.5 mM glucose. The final concentrations of K^+, Na^+, and Ca^{2+} in culture medium were 4.0 mM, 137 mM and 0.97 mM, respectively. The cell suspension was diluted to 5×10^5 cells/ml and placed in plastic Petri dishes (100 × 20 mm style, Falcon). The larger culture dishes contained 25-mm circular glass coverslips that were used in ion flux studies. Cultures were incubated in humidified 5% Co_2, 95% air atmosphere at 37°. Confluent monolayers, in which at least 80% of cells exhibited spontaneous synchronous contractions, developed by 3 days of incubation.

^{45}Ca and ^{24}Na flux and content. For measurement of ^{45}Ca or ^{24}Na uptake (20), monolayers of cells attached to glass coverslips were preincubated in HEPES buffer solution (pH 7.35) at 37° for 5 min and subsequently incubated in appropriate medium containing ^{45}Ca (5 μCi/ml) or ^{24}Na (5 μCi/ml) for the desired periods of time. Coverslips were removed from the holder in the incubation bath and quickly washed for 8 sec each in two 80-ml volumes of HEPES solution at 2–4°. Cells were then scraped off the coverslips and dissolved for 2 hr in a solution containing 1% sodium dodecyl sulfate and 10 mM sodium borate. Aliquots of solution containing dissolved cells were assayed for radioactivity and protein content.

For measurement of cellular Na content, coverslips were incubated in HEPES buffer solution (pH 7.35) containing tracer amounts of ^{24}Na (5 μCi/ml) for 30 min, at which time ^{24}Na content had plateaued. Coverslips were washed in the manner described above and cellular content of ^{24}Na was determined.

Contractility measurements. Changes in the contractile state of individual cells in the monolayers were assessed by the use of an optical-video system as previously described (19). A glass coverslip with attached monolayer of ventricular cells was continuously superfused in a chamber on the stage of an inverted phase contrast microscope with bicarbonate buffer solution (pH 7.35) containing 116.3 mM NaCl, 1 mM NaH_2PO_4, 0.8 mM $MgSO_4$, 4 mM KCl, 26.2 mM $NaHCO_3$, 0.9 mM $CaCl_2$, 5.6 mM dextrose, and 2% fetal calf serum at a rate of 2 ml/min. The pH of the buffer solution was maintained at 7.4 by continuously gassing the chamber with a 95% air, 5% CO_2 gas mixture. A constant temperature of 37° was maintained by enclosing the microscope in a thermostatted Lucite box. Following a 15-min equilibration period, cells were superfused with the same solution containing amiloride, and changes in the amplitude of motion of an individual cell were monitored.

Cell density correction. To normalize for cell density on each coverslip, the monolayers were grown in L-[4,5-^{3}H(N)]leucine (0.1 μCi/ml) for 24 hr before each experiment. ^3H counts permitted estimation of cell density on each coverslip. The relationship between radioactive counts and protein concentration allowed accurate estimation of protein concentration for each coverslip. Thus, simultaneous counting of ^3H and ^{45}Ca or ^{24}Na permitted normalization of ^{45}Ca or ^{24}Na content per mg of cell protein.

Miscellaneous. Protein concentration was assayed by the method of Lowry *et al.* (21) using crystalline bovine serum albumin as standard. Hanks' salt solution, M199, and fetal calf serum were purchased from Gibco Laboratories, Grand Island, NY. All radiolabeled compounds and ions were purchased from New England Nuclear, Boston, MA. Verapamil hydrochloride was obtained from Knoll Pharmaceutical Co., Whippany, NJ; amiloride hydrochloride was a gift from Merck, Sharp and Dohme, Rahway, NJ. All other chemicals used were of the highest grade commercially available.

Statistical analyses were performed using the Student's *t* test and two-way analysis of variance.

Results

In order to determine the effect of amiloride on contractile state, spontaneously contracting cultured chick ventricular cells were superfused with solution containing concentrations of amiloride ranging from 10 μM to 3 mM. The amplitude of cell motion, i.e., the distance that a point on the cell surface travels from the rested state to the contracted state during contraction, was monitored continuously. As shown in Fig. 1, 10 μM amiloride produced no change in the amplitude of cell motion. Higher concentrations of amiloride (0.1–0.3 mM) produced slowly developing negative inotropic effects that approached a steady state level after approximately 90 min of perfusion. A still higher concentration of amiloride (1 mM) produced a transient positive inotropic effect that lasted approximately 60 sec, and then a gradual decline in the amplitude of cell motion. By 90 min, 1 mM amiloride decreased the amplitude of cell motion to 10% of the control level. Concentrations of amiloride greater than or equal to 3 mM were associated with a faster decline in the amplitude of cell motion with no evidence of early positive inotropic effect. Amiloride also produced a time- and concentration-dependent decrease in beating rate over the range of the drug concentration used (Fig. 2). All of the effects of amiloride were reversible and could be washed out within 30–40 min by perfusion of cells with control medium.

To examine the effect of amiloride on the positive inotropic and toxic actions of ouabain, cells were superfused with concentrations of ouabain ranging from 2 μM to 6 μM in the presence or absence of 0.1, 0.3, or 1 mM amiloride. The changes in the amplitude of cell motion, development of arrhythmic beating, and contracture (shift in end-diastolic position) were then monitored. The concentration-effect curve for ouabain in the presence or absence of amiloride (1 mM) is shown in Fig. 3. The actual increase in the amplitude of cell motion was expressed as a percentage increase of the control value observed just prior to exposure to ouabain. Increasing ouabain concentrations augmented progressively the amplitude of cell motion.

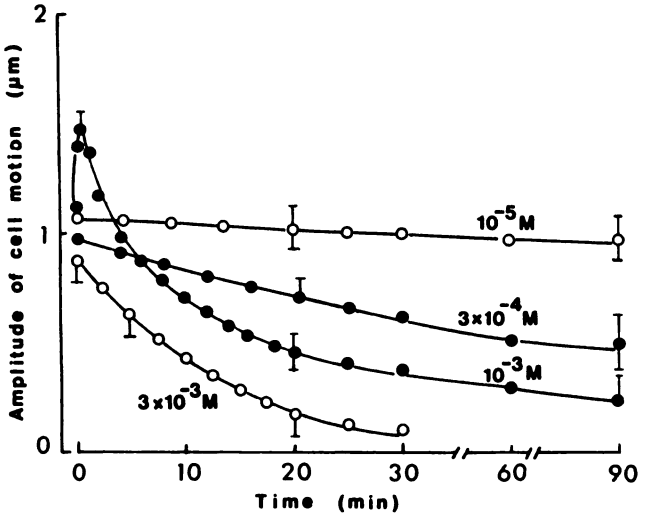

Fig. 1. Effect of amiloride on the amplitude of cell motion. Monolayers of spontaneously contracting cultured ventricular cells were prepared from 10-day-old chick embryos. The amplitude of motion of individual cells was monitored using an optical-video system (see Materials and Methods). Cells were exposed to amiloride at time zero. Each *point* represents the mean ± SE of six determinations.

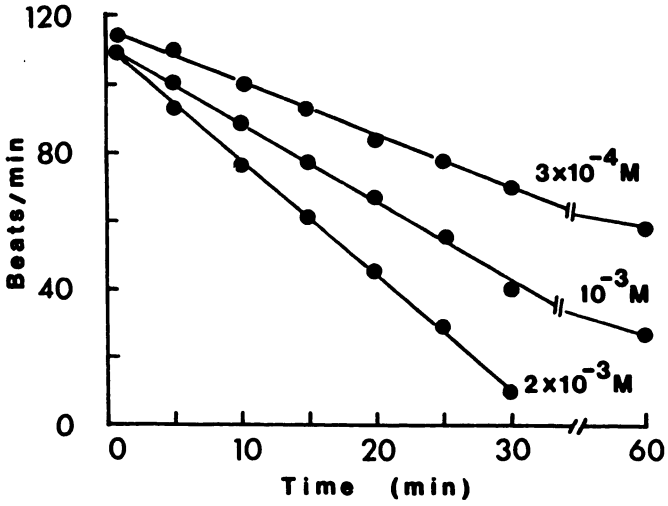

Fig. 2. Effect of amiloride on spontaneous beating rate. Cells were exposed to amiloride and the rates of spontaneous contractions were determined. Each *point* represents the mean ± SE of six determinations.

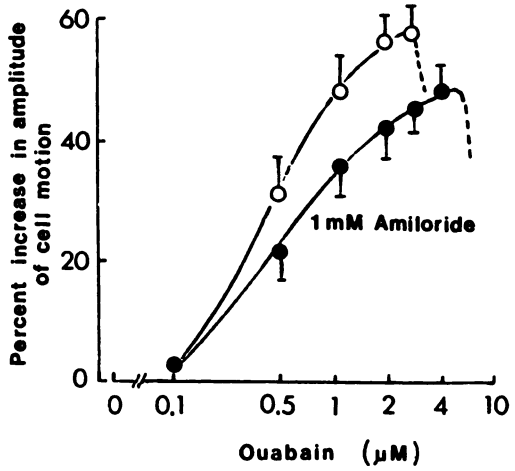

Fig. 3. Concentration-effect curve for ouabain. Cells were superfused with medium containing concentrations of ouabain ranging from 0.1 to 10 μM and changes in the amplitude of cell motion were monitored. Another group of cells was first exposed to 1 mM amiloride for 10 min and then exposed to 1 mM amiloride and ouabain. The increase in the amplitude of cell motion was expressed as a percentage increase of the amplitude observed in the absence of ouabain. Each *point* is the mean ± SE of five determinations. *Dashed lines* indicate development of rhythm disturbances.

Beginning at 3 μM ouabain, rhythm disturbances were observed (noted by the *dashed line* in Fig. 3). In the presence of amiloride (1 mM), the concentration-effect curve for ouabain was shifted to the right and the maximal increase in amplitude was also reduced. As indicated in Table 1, cells pretreated with amiloride for 10 min developed toxic responses to ouabain (arrhythmias and contracture) only at ouabain concentrations higher than those needed to produce toxic responses in control cells. This effect was amiloride concentration dependent, with higher concentrations of amiloride yielding greater protection against ouabain-induced toxicity. In general, amiloride-induced arrhythmias developed approximately 5 min after the drug exposure in all cells in which arrhythmias occurred. In one group of cells, ouabain produced arrhythmias that continued for at least 15–20 min before a slow shift in end-diastolic position indicating contracture occurred (+ in Table 1). In another group of cells, ouabain produced arrhythmias and then contracture within 5 min after the development of arrhythmias (++ in Table 1). These results indicate that amiloride reduces the sensitivity of cultured myocytes to the positive inotropic and toxic effects of ouabain.

TABLE 1

Effect of amiloride on ouabain-induced toxicity

Cells were perfused with medium containing ouabain alone or ouabain and amiloride, and the appearance of rhythm disturbance and contracture was monitored. For each group, six determinations were made. For each group, all cells developed either arrhythmias and/or contracture, or a positive inotropic effect in response to drugs. See the text for a detailed description of the time course of development of ouabain toxicity.

	Ouabain (μM)			
Amiloride (mM)	3	4	5	6
0	+[a]	+	++[b]	++
0.1	0[c]	+	+	++
0.3	0	0	+	+
1	0	0	0	+

[a] +, toxic response (arrhythmias and late contracture).
[b] ++, toxic response (arrhythmias and early contracture).
[c] 0, positive inotropic response without toxicity.

It is generally believed that the contracture (rise in end-diastolic tension) produced by exposure of cells to Na-free medium is due to an increase in $[Ca]_i$ via Na-Ca exchange (22, 23). Since cardiac glycoside-induced positive inotropic effects involve an augmentation of $[Ca]_i$ via Na-Ca exchange (19, 24–27), inhibition of this exchange by amiloride would be expected to reduce the effects of digitalis. Therefore, we examined the effect of amiloride on Na-Ca exchange by determining the magnitude of the contracture response produced by exposure to Na-free medium. Cells were superfused with control solution (0.9 mM Ca) or the same solution containing 1 mM amiloride for 15 min, and were then superfused with Na-free medium with or without 1 mM amiloride. As shown in Fig. 4A, exposure to Na-free medium resulted in the development of contracture, i.e., a shift in end-diastolic cell position with a concomitant decrease in amplitude of cell motion, followed by gradual relaxation over a 2–4-min period. The presence of 1 mM amiloride was associated with a slightly but significantly reduced magnitude of the contracture signal [−18 ± 3% (mean ± SE), $p < 0.05$] compared to that in control cells. No changes in relaxation properties were evident. These results suggest that amiloride decreases the accumulation of $[Ca]_i$ via Na-Ca exchange, as manifest by reduced contracture.

Since amiloride reduced cellular Na content by 16% (from 57 to 48 nmol/mg of protein; see below), it seemed likely that the amiloride-induced reduction in zero $[Na]_o$-induced contracture was related to the lower cellular Na content and, therefore, reduced Ca accumulation via Na-Ca exchange. To test this hypothesis, we lowered cellular Na content by preincubation of cells in medium that contained 115 mM Na (ionic strength maintained constant with choline) for 30 min. This procedure lowered cellular Na content by 17% from control. When cells treated in this way were abruptly exposed to Na-free medium, the magnitude of the contracture signal that developed was indistinguishable from the contracture produced in the presence of 1 mM amiloride (Fig. 4B). These results are consistent with the view that amiloride reduced the magnitude of the zero $[Na]_o$ contracture as a result of the decreased cellular Na content, rather than as a result of direct inhibition of Na-Ca exchange. Thus, amiloride appears to affect Ca accumulation

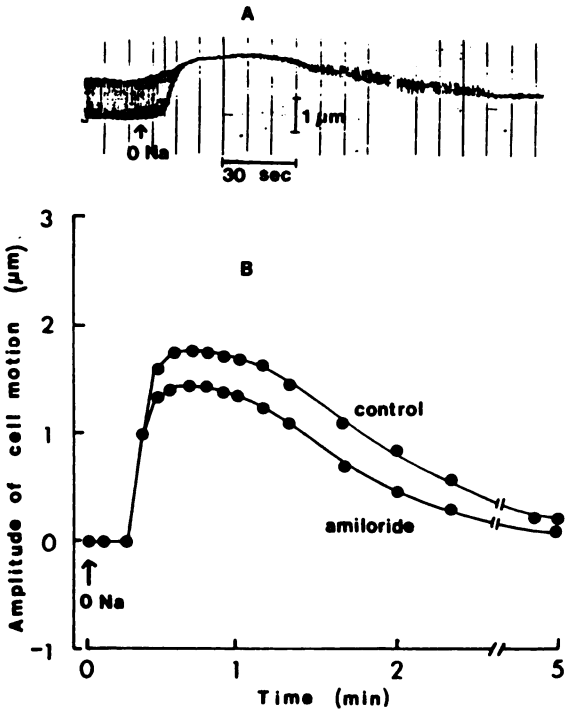

Fig. 4. Zero $[Na]_o$-induced contracture in cultured chick ventricular cells. Cells were equilibrated in HEPES-buffered medium (pH 7.4) containing 140 mM Na and then abruptly exposed to medium containing no Na and 140 mM choline. A shows the development of contracture induced by zero Na medium, and subsequent relaxation (typical tracing). B shows plots of the changes in end-diastolic position in the presence and absence of 1 mM amiloride. Each *point* is the mean ± SE of six experiments. The two *curves* are significantly different ($p < 0.05$).

via Na_i-dependent Ca uptake as a consequence of its primary effect on intracellular Na content.

Ca fluxes. To test directly the effect of amiloride on Ca influx and the magnitude of the rapidly exchangeable Ca pool, ^{45}Ca uptake by myocyte monolayers was measured at selected time intervals. Cells were preincubated in medium containing 1 mM amiloride for 10 min and then incubated in medium containing 1 mM amiloride and ^{45}Ca. ^{45}Ca uptake in control cells was also measured in the absence of amiloride. ^{45}Ca uptake during the initial 5-min period consisted of an early rapid phase that was complete by 1–2 min and a plateau phase (5 min) that defines the rapidly exchangeable Ca pool (20). Amiloride reduced significantly both the initial rate of ^{45}Ca uptake and the ^{45}Ca content at 5 min (Fig. 5). As shown, the addition of 1 μM verapamil to preincubation and incubation media did not prevent the effect of amiloride on ^{45}Ca uptake. Since Ca influx occurs mainly via slow Ca channels and Na-Ca coupled exchange, these results suggest that amiloride selectively reduced Ca influx via the Na_i-dependent Ca uptake pathway.

To examine further the effect of amiloride on Ca influx via Na-Ca exchange, cells were exposed first to 1 mM ouabain to load the cells with Na and 1 μM verapamil to inhibit Ca flux via slow Ca channels. The cells were then exposed to Na-free medium containing ^{45}Ca. Preexposure to 1 mM ouabain markedly increased the initial rate of ^{45}Ca uptake (Fig. 6). The additional presence of 1 μM verapamil reduced the rate of ^{45}Ca uptake by only 10% compared to the response to 1 mM ouabain alone. The increase in Ca uptake produced by ouabain is presumably due to enhanced Na_i-dependent Ca influx, which is markedly stimulated by an elevated concentration of intracellular Na and absence of extracellular Na. Under these conditions, 1 mM amiloride caused a partial inhibition (40%) of the initial rate of ^{45}Ca uptake. These results show that high amiloride concentrations reduce Ca influx via Na-Ca exchange, consistent with the previously described effects of amiloride on the development of contracture induced by Na-free medium.

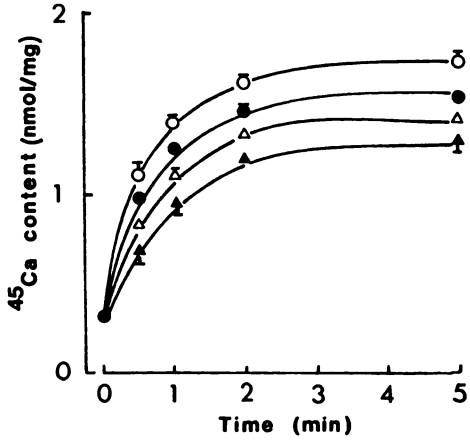

Fig. 5. Effect of amiloride on ^{45}Ca uptake. Cells were preincubated in medium containing no drug (○), 1 mM amiloride (●), 1 μM verapamil (△), or 1 mM amiloride plus 1 μM verapamil (▲) for 10 min and then incubated in the same medium containing ^{45}Ca. The amount of ^{45}Ca taken up by the cells was then assayed. Each *point* represents the mean ± SE of eight determinations. All *curves* are significantly different from the corresponding control curves ($p < 0.05$).

Under the conditions described, amiloride reduced the ouabain (1 mM)-induced elevation of cellular Na content by 45% (see below). We therefore tested the hypothesis that the 40% reduction in the initial rate of ^{45}Ca uptake produced by amiloride was caused by the lower cellular Na content. Cells were preincubated in medium containing 70 mM Na, 50 mM choline, and 1 μM verapamil to mimic the effect of amiloride. This procedure lowered mean cellular Na content to 58% of control (159–92 nmol/mg of protein). The initial rate of ^{45}Ca uptake by these Na-depleted cells was not significantly different from that observed in amiloride-treated cells (Fig. 6). These results are consistent with the view that amiloride does not affect Na-Ca exchange directly but, rather, reduces the exchange rate by lowering cellular Na content. Also shown in Fig. 6 is the response to lanthanum, an inhibitor of Ca influx via Na-Ca exchange as well as slow Ca channels (20), which abolished the effect of ouabain on ^{45}Ca uptake as well as the total Ca uptake.

To determine whether the amiloride-induced decrease in sensitivity to the toxic effects of ouabain correlated with reduced Ca influx, we next examined the effect of amiloride on the ouabain-induced increase in Ca influx. Cells were preexposed to medium containing concentrations of ouabain ranging from 0 to 6 μM with or without 1 mM amiloride for 30 min. Cells were then incubated in the identical medium except that it contained ^{45}Ca and no Na^+ (Na was replaced by choline), and the initial rates of ^{45}Ca uptake were determined. As shown in Fig. 5, amiloride in the absence of ouabain decreased the mean initial rate of ^{45}Ca uptake by 12% ($p < 0.05$). Addition of increasing concentrations of ouabain caused progressive increases in the initial rate of ^{45}Ca uptake (Fig. 7) in the presence or absence of amiloride. In the presence of 1 mM amiloride, however, the increment in ^{45}Ca uptake produced by a given concentration of ouabain was significantly reduced compared to the corresponding value in the absence of amiloride. Partic-

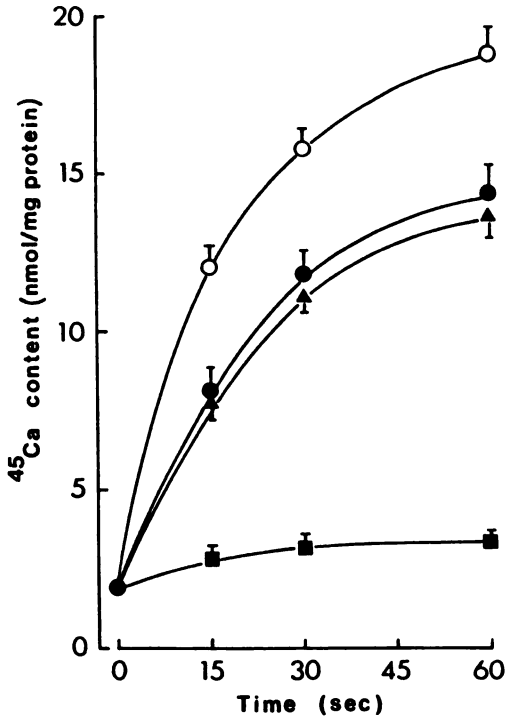

Fig. 6. Initial rates of ^{45}Ca uptake. Cells were preincubated in medium containing 140 nM Na, 1 mM ouabain, and 1 μM verapamil with (●) or without (○) 1 mM amiloride for 10 min. The cells were then incubated in Na-free medium (Na replaced with choline) containing ^{45}Ca and the same combinations of drugs. In another group of cells (▲), cells were preincubated in medium containing 70 mM $[Na]_o$, 1 mM ouabain, and 1 μM verapamil. In another group of cells, 1 mM lanthanum chloride was added to both media (■). Each *point* is the mean ± SE of eight determinations. All values are significantly different from the corresponding control values ($p < 0.01$).

ularly pronounced differences in ^{45}Ca uptake rate were apparent at toxic concentrations of ouabain. These data provide direct evidence that amiloride blunts ouabain's ability to enhance Ca accumulation via Na-Ca exchange.

Na flux and content. It is well documented that amiloride inhibits Na-H exchange in myocardial cells (14, 15). To investigate the effect of amiloride on cellular Na content and Na flux via Na-H exchange, cells were preincubated in medium with 1 mM amiloride for 30 min and then incubated in medium with 1 mM amiloride, 1 mM ouabain, and ^{24}Na. In the absence of amiloride, the rate of ^{24}Na uptake was linear up to 20–30 sec (Fig. 8). The addition of 1 mM amiloride to the media did not alter significantly the initial rate of ^{24}Na uptake (0–20 sec). Subsequently, however, amiloride reduced the rate of ^{24}Na uptake (30–60 sec) such that ^{24}Na contents at these times were significantly lower than corresponding ^{24}Na contents in control cells. By 30 min, mean cellular Na content was 45% lower in amiloride-treated cells than in control cells (172 versus 95 nmol/mg of protein). The large percentage decrease in ^{24}Na content as 30 min caused by amiloride (see Fig. 8, *inset*) was probably due to the effect of 1 mM ouabain that was present in the uptake medium. As shown in Fig. 9, amiloride produced a graded reduction of ^{24}Na uptake and content with increasing concentrations of ouabain. The initial Na influx rate, as might be expected, was significantly decreased by 1 μM TTX and 1 μM verapamil, which block fast Na channels and slow Ca channels, respectively, and cause cessation of spontaneous beating of cultured myocytes. Further addition of amiloride produced no measurable decrease in ^{24}Na uptake until 30 sec of uptake. The remaining portion of ^{24}Na uptake that was not inhibited by TTX and verapamil was probably due at least in part to Na-Na exchange, which has been reported to occur in several types of cells including cardiac myocytes (28–30). Since the initial rate of ^{24}Na uptake was not influenced significantly by amiloride, these findings suggest that Na influx via amiloride-sensitive pathways (i.e., Na-H exchange) does not consti-

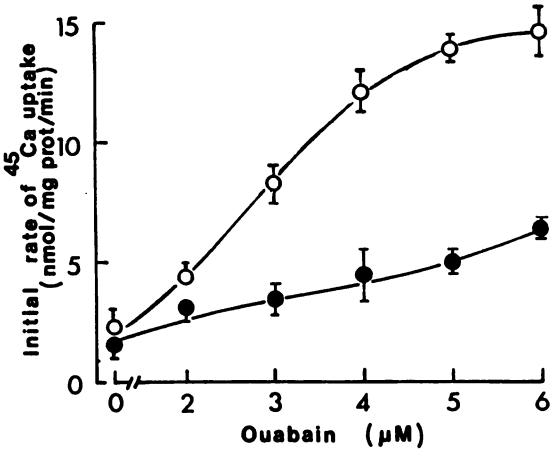

Fig. 7. Effect of amiloride and ouabain on the initial rate of ^{45}Ca uptake. Cells were preincubated for 10 min in HEPES-buffered medium (pH 7.4) containing concentrations of ouabain ranging from 0 to 6 μM in the presence (●) or absence (○) of 1 mM amiloride. The cells were then incubated in Na-free medium containing ^{45}Ca and the same combinations of drugs for 30 sec. Each *point* is the mean ± SE of eight determinations. All values observed in the presence of amiloride are significantly lower than the corresponding values observed in the absence of amiloride ($p < 0.01$).

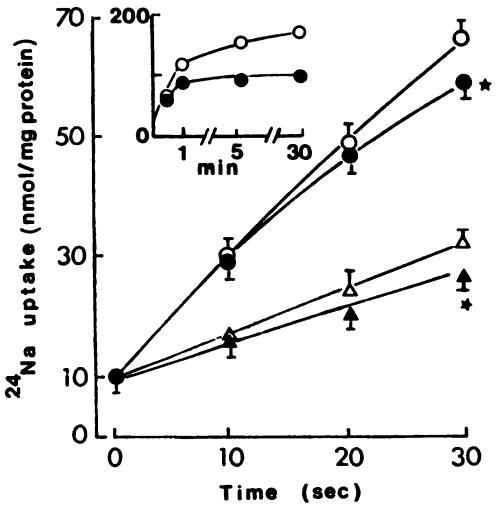

Fig. 8. Effects of amiloride, TTX, and verapamil on ^{24}Na uptake. Cells were equilibrated in HEPES-buffered medium (pH 7.4) and then incubated in medium containing ^{24}Na and 1 mM ouabain (○). In other groups of cells, 1 mM amiloride (●), 1 μM TTX and 1 μM verapamil (△), or 1 mM amiloride, 1 μM TTX, and 1 μM verapamil (▲) were added to the equilibration (10 min) and incubation media. The *inset* shows the full time course of ^{24}Na accumulation for the control and amiloride-treated cells. Each *point* is the mean ± SE of eight determinations. ★, significantly different from the corresponding control values ($p < 0.05$).

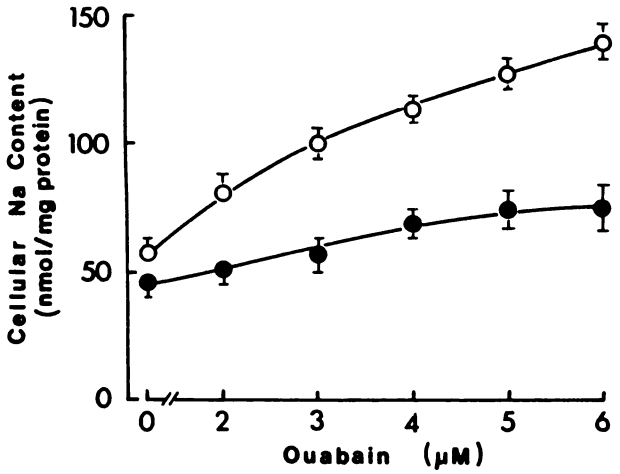

Fig. 9. Effect of ouabain and amiloride on cellular Na content. Cells were incubated to steady state (30 min) in medium containing ^{24}Na and concentrations of ouabain ranging from 0 to 6 μM in the presence (●) or absence (○) of 1 mM amiloride. Cellular Na content was determined by assaying the amount of ^{24}Na in the cell. Each *point* is the mean ± SE of 10 determinations. All values observed in the presence of amiloride are significantly lower than the corresponding values observed in the absence of amiloride ($p < 0.05$)

tute a substantial contribution to the total Na influx observed in cultured chick ventricular cells under physiologic conditions.

Although amiloride reduced cellular Na content by 16% (57 ± 4 to 48 ± 3 nmol/mg of protein), this reduction was not due to decreased beating rate produced by amiloride since alterations of beating rate by exposure of cells to various concentrations of $[K]_o$ did not alter significantly the cellular Na content. For example, cells exposed to 2.5 mM and 4 mM $[K]_o$ had beating rates (per min) of 120 ± 3 and 79 ± 4, and cellular Na contents of 58 ± 6 and 52 ± 4 nmol/mg of protein, respectively. Amiloride also reduced the ouabain (1 mM)-induced increase in intracellular Na concentration. Since the direct effects of ouabain on the heart are mediated largely through the intracellular Na concentration, it follows that altered sensitivity of cultured heart cells to toxic actions of ouabain are probably due to reduced cellular Na content and, consequently, diminished Ca accumulation via Na-Ca exchange (31). To test this hypothesis, cellular Na content was determined in cells exposed to concentrations of ouabain ranging from 2 μM to 6 μM in the presence or absence of 1 mM amiloride. As shown in Fig. 9, increasing concentrations of ouabain over this range were associated with increasing cellular Na content. In the presence of 1 mM amiloride, the increase in Na content produced by a given concentration of ouabain was significantly reduced compared to that observed in the absence of amiloride. The difference in cellular Na content observed with and without amiloride became progressively larger with higher ouabain concentrations. These results show that amiloride alone only slightly reduces the cellular Na content but produces more marked inhibition of the ouabain-induced elevation of cellular Na content. Therefore, the reduced sensitivity of cells treated with amiloride to toxic effects of ouabain can be accounted for fully by the effect of amiloride on cellular Na content.

To investigate the mechanism by which amiloride causes greater reduction of cellular Na content in the presence of ouabain, Na uptake rates were determined. Cells were preincubated in medium containing 3 μM ouabain for 10 min and then exposed to medium containing ^{24}Na and 1 mM ouabain to block Na efflux with or without 1 mM amiloride. As shown in Fig. 10, amiloride caused a marked inhibition of the initial rate of ^{24}Na. This is in contrast to the effect observed in the absence of ouabain pretreatment, in which only a small reduction in ^{24}Na uptake was present. With longer exposure periods, the differences in ^{24}Na content became similar to that shown in the *inset* of Fig. 8, probably as a result of the effect of 1 mM ouabain that was present in the uptake medium to prevent Na efflux. These results indicate that amiloride reduces cellular Na content to a greater degree in the ouabain-treated cells than in control cells, by inhibition of Na influx via the amiloride-sensitive pathway.

Effects of intracellular pH. Amiloride may antagonize the effects of ouabain by altering the pH_i of the cell, since amiloride has been shown to cause intracellular acidification by 0.2 pH units in sheep heart Purkinje fibers. To test this possibility, we transiently lowered pH_i using NH_4Cl and determined the effect of ouabain on cellular Na content under normal and reduced pH_i conditions. Cells were incubated in medium containing ^{24}Na for 30 min (steady state) in the presence or absence of 20 mM NH_4Cl during the last 5 min of incubation. The cells were then incubated further for 5 min in medium containing ^{24}Na with the same specific activity and no NH_4Cl with or without 1 mM ouabain. Addition and subsequent removal of NH_4Cl has been shown to cause intracellular acidification (15). Exposure of cells to 20 mM NH_4Cl reduced cellular Na content by 12% (56 ± 5 to 49 ± 3 nmol/mg of protein) and subsequent removal of NH_4Cl augmented cellular Na content by 26% (56 ± 5 to 70.5 ± 4 nmol/mg of protein). Ouabain (1 mM) elevated cellular Na content to similar extents in normal or NH_4Cl-treated cells (280 ± 18 versus 262 ± 15%). Thus, a decrease in pH_i by itself did not interfere with the ability of ouabain to increase cellular Na content, in contrast to the effect of amiloride which markedly inhibited ouabain-induced augmentation of cellular Na content.

Discussion

Amiloride is a potassium-sparing diuretic drug that has been reported to have several important effects on cardiac muscle,

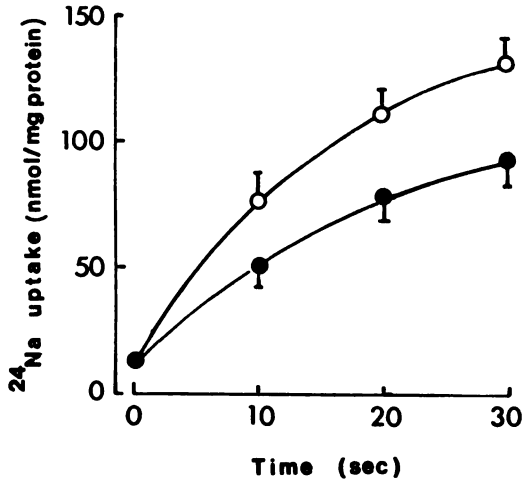

Fig. 10. Effect of amiloride on ^{24}Na uptake in ouabain-treated cells. Cells were preincubated in HEPES-buffered medium (pH 7.4) containing 3 μM ouabain for 10 min and then exposed to medium containing 1 mM ouabain and ^{24}Na with (●) or without (○) 1 mM amiloride. Each *point* is the mean ± SE of nine determinations. All values observed in the presence of amiloride are significantly lower than the corresponding values observed in the absence of amiloride ($p < 0.05$).

including reduction of the toxic effects of cardiac glycosides (2). We have examined the direct effects of amiloride on the contractile state, beating rate, and Na and Ca movements across the sarcolemmal membrane of control and ouabain-treated cultured chick ventricular cells. Our results show that at relatively high concentrations (1 mM), amiloride has pronounced negative inotropic and chronotropic effects accompanied by significant reductions in sarcolemmal Ca and Na fluxes and cellular Ca and Na contents. The changes in Ca flux and content appear to be secondary to reduced Na flux and content via Na-H exchange produced by amiloride. These changes in ion movements across the sarcolemmal membrane account, at least in part, for the protection by amiloride from cardiac glycoside-induced toxicity.

Amiloride has been shown to cause a concentration-dependent prolongation of action potential duration and lengthening of the refractory period (3, 8), and also a positive inotropic effect in isolated guinea pig atrial muscle (6, 32). Berlin *et al.* (13), however, reported that, in guinea pig left atrial muscle, amiloride produced an initial positive inotropic effect followed by a return toward the control level. Our results show that 1 mM amiloride produced an early transient increase in the amplitude of cell motion that lasted only 60 sec, followed by a gradual decrease to a level markedly below control. At higher (3 mM) or lower (0.3 mM) concentrations, amiloride had only a negative inotropic effect. These results suggest that the effect of amiloride on the contractile state is quantitatively and, perhaps also qualitatively, species-dependent.

Amiloride has been reported to increase the doses of digitalis required to produce positive inotropic and toxic effects, to reverse the electrocardiographic changes induced by digitalis, and to delay the onset of arrhythmias (4, 5) in humans as well as in experimental animals. In isolated guinea pig atrial muscle preparations, amiloride also delayed the onset of digoxin-induced arrhythmias (7) and abolished the ouabain-induced rise in end-diastolic tension (6). In cultured chick heart cells, which are devoid of neural input or endogenous neuroeffectors, we observed similar antagonism of the positive inotropic and toxic (arrhythmias and contracture) actions of ouabain by amiloride. This supports the view that direct effects of amiloride on the cardiac cell are involved in the observed digitalis-amiloride antagonism. The concentrations of amiloride that we have used in this study are presumably much higher than plasma levels that occur clinically, and caution must be used in extrapolating results. However, the directionality of the effect of amiloride (i.e., antagonism of glycoside-induced increase in $[Na]_i$) is likely to be the same. Although high, the amiloride concentrations that we used did not produce arrhythmias or alter resting tension during the experimental periods.

Recently, several studies have focused on the cellular mechanism of amiloride-digitalis interaction. In purified sarcolemmal vesicles prepared from beef heart, the initial rate of Na^+-dependent Ca^{2+} uptake into Na^+-loaded vesicles was inhibited by amiloride (IC_{50}, 0.35 mM) (6). Altschuld *et al.* (30) also presented evidence for the inhibition of Na-Ca exchange across the sarcolemma of rat cardiac myocytes by amiloride. In the latter study, 10 mM amiloride inhibited the initial rapid uptake of ^{45}Ca by Na-loaded myocytes but accelerated the net uptake of Ca by myocytes with normal $[Na^+]_i$ levels. However, Frelin *et al.* (14) showed that, in cultured chick heart cells, the amiloride-sensitive $^{22}Na^+$ flux component was independent of the presence of $[Ca^{2+}]_o$ and concluded that amiloride had no direct effect of Na-Ca exchange. In cultured chick ventricular cells, we report here that amiloride caused a significant inhibition of the initial rate of ^{45}Ca uptake. However, this proved to be due entirely to the reduction in cellular Na content produced by amiloride pretreatment, and not to a direct action of amiloride on Na-Ca exchange. These results are in agreement with the findings of Frelin *et al.* (14).

The rise in end-diastolic tension (contracture) induced by exposure of cells to Na-free medium is generally thought to be due to Ca influx via Na-Ca exchange and consequent Ca overload (22, 33). When cells were equilibrated in 140 mM medium and then abruptly exposed to Na-free medium (Na replaced by choline) in the continuous presence of 1 mM amiloride, the amplitude of the contracture signal was 18% less than that without amiloride, suggesting that amiloride slightly reduced the Ca accumulation via Na-Ca exchange. However, we noted that 1 mM amiloride reduced cellular Na content by 16% under identical circumstances. To test whether the amiloride-induced reduction in contracture could be accounted for by decreased $[Na]_i$, cellular Na content was first lowered to 83% of control level by preincubation of cells in 115 mM Na (choline-substituted) medium. Subsequent exposure of cells to Na-free medium in the absence of amiloride resulted in an 18% decrease in contracture magnitude compared to that observed in cells preincubated in 140 mM Na. These findings indicate that amiloride reduces Ca accumulation via Na-Ca exchange as a consequence of reduced $[Na]_i$. Thus, amiloride might be expected to antagonize the action of digitalis by lowering $[Na]_i$ and consequently reducing Ca accumulation via Na-Ca exchange.

Ouabain at 4 μM produced rhythm disturbances and contracture in control cells, but yielded only a positive inotropic effect in amiloride-treated cells. This concentration of ouabain produced a 4.5-fold increase in the initial rate of ^{45}Ca uptake in control cells in response to a step change to zero $[Ca]_o$, but only a 2.6-fold increase in amiloride-treated cells. The reduced Ca uptake rate in the presence of amiloride can be wholly explained by the diminution of cellular Na content. When cellular Na content was comparably reduced by preincubation in low Na medium (70 mM Na) such that the cellular Na content was similar to that observed in amiloride-treated cells, the initial rates of ^{45}Ca uptake were indistinguishable in the two groups of cells. Thus, these results are entirely consistent with the view that the antagonism by amiloride of ouabain-induced toxicity is due to the reduction of intracellular Na concentration produced by amiloride, presumably by inhibition of Na-H exchange.

Our results show that 1 mM amiloride reduced cellular Na content by a mean value of 16%. This is in good agreement with the findings of other investigators, who found a 13–16% decrease in $[Na]_i$ (14–16). Frelin *et al.* (14) also reported that amiloride inhibited ^{22}Na uptake by cultured chick heart cells, and suggested that this was due to inhibition of Na-H exchange. These investigators found that 0.1 mM amiloride caused a 50% or greater reduction in ^{22}Na uptake and, therefore, concluded that the Na-H exchange system is a major uptake pathway for Na. However, the ^{22}Na uptake experiments were performed in 3 mM Na medium following preexposure of cells to Na-free medium. These manipulations may favor Na influx via Na-Ca exchange as well as Na-H exchange, since $[Na]_i$ presumably

was markedly reduced and $[Ca]_i$ may have been increased. Therefore, it is not clear what fraction of ^{22}Na uptake was due to Na-H exchange under physiologic conditions. The results shown in Fig. 7 indicate that under physiologic conditions, amiloride causes much less inhibition of ^{24}Na uptake. This suggests that Na influx via Na-H exchange does not constitute a major fraction of total Na flux under normal conditions. However, when Na-H exchange is stimulated (for example, by intracellular acidification). Na influx via Na-H exchange has been shown to be markedly augmented (15, 29, 34). Although a major component of steady state Na-H exchange could not be detected from ^{24}Na uptake experiments, the finding that amiloride reduced cellular Na content significantly is consistent with the view that Na-H exchange does occur under normal conditions. Other investigators also have not found appreciable Na-H exchange in cultured skeletal muscle cells (35) or thymic lymphocytes (36) under normal culture conditions. Moolenaar *et al.* (37), however, detected a small steady state rate of Na-H exchange in neuroblastoma cells. Thus, the magnitude of steady state Na-H exchange appears to be tissue specific.

In contrast to the small effect of amiloride on Na uptake rate under normal conditions, amiloride produced a substantial decrease in the initial rate of ^{24}Na uptake when cells were preexposed to ouabain. This indicates that inhibition of Na influx via the amiloride-sensitive pathway is greater in the presence of ouabain than in its absence, and may explain the greater inhibitory effect of amiloride on cellular Na content when ouabain is present. Studies with more potent inhibitors of Na-H exchange such as ethylisopropylamiloride have indicated that ouabain-induced elevation of cellular Na content can be markedly reduced by this Na-H exchange inhibitor (14). These findings are consistent with our results that the amiloride-induced decrease in cellular Na content in ouabain-treated cells is large, and support our view that Na-H exchange is involved.

Amiloride may antagonize the effects of ouabain by altering the pH_i of the cell, since amiloride has been shown to cause intracellular acidification by 0.2 pH units in sheep heart Purkinje fibers (16). In additional experiments we used NH_4Cl to reduce pH_i by at least 0.2–0.3 pH unit (15, 16) and examined the effect of ouabain on cellular Na content under normal or reduced pH_i conditions. During transient intracellular acidification produced by exposure to and subsequent removal of NH_4Cl, 1 mM ouabain elevated cellular Na content to similar extents in normal or NH_4Cl-treated cells. This is in contrast to the effect of amiloride, which markedly inhibited ouabain-induced augmentation of cellular Na content. Reduction of pH_o from 7.4 to 6.4, which has been shown to cause a pH_i fall of 0.2 unit in sheep heart Purkinje fibers (16), also failed to alter significantly the positive inotropic and toxic effects of ouabain, provided that cellular Na content was maintained at the control level. These results support the view that amiloride antagonizes the effect of ouabain on cellular Na content by inhibition of Na-H exchange and not by reduction of pH_i of the magnitude expected in this study.

In summary, we studied the effect of amiloride and ouabain, alone and in combination, on contractile state and on Na and Ca fluxes and contents in spontaneously contracting cultured chick ventricular cells. Amiloride had no measurable direct effect on Na-Ca exchange but reduced the Ca accumulation via Na-Ca exchange as a consequence of reduced cellular Na content. Amiloride (1 mM) produced a transient positive inotropic effect followed by a gradual negative inotropic effect over 60 min, with concomitant gradual decrease in beating rate. Amiloride antagonized the positive inotropic and the toxic effects of ouabain by marked inhibition of amiloride-sensitive Na uptake (Na-H exchange) and of the ouabain-induced elevation in cellular Na content, and, consequently, of Ca accumulation via Na-Ca exchange.

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